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Comparative Study on LDH Isozymes in Different Subfamily of Teleost Fish - Grass Carp (*Ctenopharyngodon idellus*) and Blunt Snout-Bream (*Megalobrama amblycephala*)

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ABSTRACT—Electrophoretic, physico-chemical and immuno-analysis of lactate dehydrogenase isozymes of grass carp (*Ctenopharyngodon idellus*) and blunt snout-bream (*Megalobrama amblycephala*) indicated that although they were rather conservative in evolution, however, some divergences in their gene activities and molecular structures were still remained. So, LDH isozyme can be used as a genetic marker to distinguish these two kinds of fish.

INTRODUCTION

Markert and Faulhaber [1], using starch gel electrophoresis, examined the LDH isozyme patterns in 30 kinds of fish. They found out that the LDH isozymes in fish were more complicated than those in mammals and birds. One to twenty LDH isozymes were found in different fish examined. They were found distributed differently in various tissues in different kinds of fish and were classified into three systems. Among them a major LDH isozyme system was distributed in most tissues corresponding to the A and B gene systems of mammals and birds. Two minor systems were restricted to eye and gonads. Through electophoresis and immuno analysis, Shaklee *et al.* [2] found that the two minor systems were probably coded by the same gene locus, corresponding to the C gene of mammals and birds. In comparison of the kinetic properties, amino acid content and electrophoretic zymograms of LDH isozymes of Brook trout, lake trout and their hybrid, Splake trout, Wuntch and Goldberg [3] found out that there were some differences of all these properties. Yan *et al.* [4] reported that the electrophoretic zymograms of LDH isozymes can be used for distiguishing four kinds of subfamily teleost fish in the Family *Cyprininae*. This means that the LDH isozymes not only behave differently in tissue distributions, but also could be performed as a species-specific marker in fish taxonomy.

For accurate comparative study of isozymes in different organisms, purified LDH isozyme fractions must be obtained. In 1967, Okabe *et al.* [5] seperated and purified five human LDH isozyme fractions by using the ammonium sulfate fractionation, calcium phosphate gel absorption and DEAE-cellulose separation methods. In 1970, Cuatrecasas [6] using a very simple and rapid method—blue dextran affinity chromatography separated and purified human LDH 1 and LDH 5. Later, Fulton *et al.* [7] using HPLC separated five LDH isozyme fractions in rat. But up to now, the report about the LDH isozymes separation in fish has not been seen.

In this investigation, we have tried to purify LDH isozyme and analyze their divergences in respect to their electrophoretic zymograms, immuno-properties, molecular structures as well as physico-chemical properties in two kinds of fish which belong to a different subfamily—grass carp and blunt snout-bream. Since it has also been known that the LDH 1 is a gene B product and sometimes the divergence of gene B is larger than

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gene A [8], so, the LDH 1 was preferentially to use in this investigation.

MATERIALS AND METHODS

1. Experimental animals: Grass carp (*Ctenopharyngoden idellas*) and blunt snout bream (*Megalobrama amblycephala*) were used for these experiments. Both of them belong to the same family (*Cyprininae*) but to different subfamilies and genera. Grass carp belongs to the Subfamily *Leucinae* and blunt-snout bream belongs to the Subfamily *Abramidinae*. They were purchased from Wan Quan Zhuang Fishery Farm, Beijing, and were two years old.

2. Chemicals: Blue dextran and sepharose 4B were purchased from Pharmacia Chemical Co., Sweden; Nitro blue tetrazolium (NBT) and Phenazine methosulfate (PMS) from Buchs Chemical Co., Switzerland; Starch (Lot 387–1) from Cannaught Laboratories Limited Co., Canada; NADH from Boehringer Mannheim Chemical Co., Western Germany: NAD from Yeast Plant of Shanghai, China.

3. Preparation of tissue extracts: Tissues were taken from freshly killed fish, washed with cold 0.75% saline, then homogenized and centrifuged at 15,000 rpm (MSE-18) for 30 min. The supernatants were used for starch electrophoresis.

4. Preparation of blood samples: blood was collected from caudal vein, washed with 0.75% saline containing heparin three times. The red blood cells were hemolysed with 2 ml double distiled water, then centrifuged at 3,000 rpm (k 70, Eastern Germany). The supernatants were used for electrophoresis and affinity chromatography analysis.

5. Electrophoresis and specific stain: Vertical starch gel electrophoresis and LDH isozymes staining were carried out following Xue's improved method [9]. The starch gel electrophoresis was carried out at 4°C for 16 hr. And then the gels were immersed in a specific staining solution at 37°C for 1–2 hr. Every 100 ml staining solution contains 50 mg NAD, 30 mg NBT, 2 mg PMS, 15 ml 0.5 M Tris-HCl buffer, pH 7.2, 10 ml 1 M sodium lactate, and 5 ml 0.1 M NaCl.

6. Purification of LDH: LDH 1–5 and LDH 1 were purified by blue dextran affinity chromatography mainly following Cuatrecasas' method [6] and only the NAD and NADH concentrations of the eluted buffer were changed. The elution buffer for LDH 1–5 was 0.35 mg/ml of NADH, 10 mM of Tris, 0.5 mM of mercaptoethanol, pH 8.6. The elution buffer for LDH 1 was 0.05 mg/ml of NAD, 0.1 mg/ml of lithium lactate, 10 mM of Tris, 0.5 mM mercaptoethanol, pH 8.6.

7. Assay for LDH activity and purity: LDH activity was determined spectrophotometrically by monitoring the formation of NADH at 340 nm in 1 cm quartz cuvettes following the procedure of Holmes *et al.* [10]. LDH purity was determined by enzyme specificity staining with the starch gel, and by measuring the international units (I.U.) per milligram of the extracted LDH. LDH concentration was determined as protein concentration by the method of Lowry *et al.* [11].

8. Amino acid content assay: About 0.5 mg of enzyme were hydrolyzed in 6 N HCl at 110°C for 24 hr. The resultant hydrolysate was washed and evaporated to dryness and then resuspended in 0.2 ml of double distilled water. Amino acid content was determined by HPLC (Waters Company, Model AAA) according to the ion exchange separation method described in Waters Associates Operator's Manual [12].

9. Kinetic parameter determination: The Km value of LDH 1 was calculated from Lineweaver-Burk plots, with sodium lactate as substrate at pH 8.6 and temperature 25°C. Kinetic of heat activation: The LDH 1 activity was measured at the temperatures ranging from −4°C to 80°C, at pH 8.6. Kinetics of acid and alkali treatment: The LDH 1 activity was measured in the pH range of 6 to 13, at temperature 25°C.

10. Preparation of antibody and investigation on LDH immuno properties: Rabbits were immunized with purified LDH 1–5 isozymes prepared from blunt snout-bream red blood cells following the method of Clausen [13]. A mixture of grass carp and blunt snout-bream LDH 1–5 isozymes and antisera were incubated for 30 min at 30°C prior to starch gel electrophoresis and LDH staining for neutralization or inhibition tests. The immunoprecipitated bands left in the double immuno diffusion agar gels were oberved.

RESULTS

LDH isozymes electrophoresis zymograms

The zymograms of LDH isozymes from different tissues of grass carp and blunt snout-bream were displayed by starch gel electrophoresis and they are shown in Figure 1a and 1b. Among them, in the tissues of cardiac muscle, skeletal muscle and eye of both kinds of fish, there existed five LDH isozyme bands, migrating towards the anode, and in liver, there was the C band migrating towards the cathode in both kinds of fish. In kidney tissue there were still five bands in the sample of blunt snout-bream, but seven bands in that of grass carp. The two 'additional bands' also migrated towards the anode in between LDH 2 to LDH 3 and LDH 3 to LDH 4.

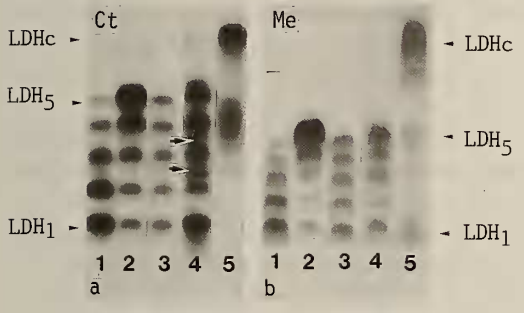

FIG. 1. LDH isozyme zymograms of various tissues of grass carp(Ct) and blunt snout-bream(Me) revealed by starch gel electrophoresis. Ct(a), Me(b). 1. Cardiac muscle, 2. Skeletal muscle, 3. Eye, 4. Kidney, 5. Liver. Arrows show 2 additional bands.

Purification of LDH 1–5 and LDH 1 from blood cells

The optimum concentration of NADH for separating LDH 1–5 was 0.39 mg/ml and the optimum concentration of NAD for separating LDH 1 is 0.05 mg/ml. Figure 2a and 2b shows the purified LDH 1–5 and LDH 1 isozymes of grass carp and blunt snout-bream. The specific activities of these purified LDH isozymes were shown in Table 1 by calculating the international units (I.U.) per milligram for purified LDH protein. The data indicated that all the purified LDH isozymes were over 500 I.U. per milligram.

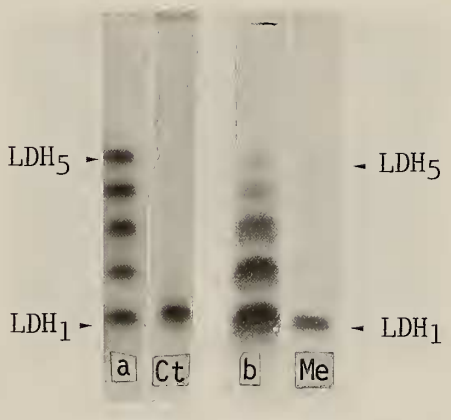

FIG. 2. Purified LDH 1–5 (left) and LDH_1 (right) isozyme zymograms of red blood cell of grass carp(Ct) and blunt snout-bream(Me) revealed by starch gel electrophoresis. Ct(a), Me(b).

TABLE 1. The specific activities of LDHl-5 and LDHl isozymes extracted and purified from the red blood cells of grass carp (Ct) and blunt snout-bream (Me)

	I.U./mg △O.D. 340×20*
Fish LDH	6.2 m
Ct LDHl-5	510
Me LDHl-5	1035
Ct LDHl	515
Me LDHl	1225

* Average value of three times of measurement.

Immuno properties

The antiserum against blunt snout-bream red blood cell LDH 1–5 isozyme were used. After the double diffusion on agar gel with blood sample of grass carp and blunt snout-bream, a complete crossing precipitation line was clearly observed (Fig. 3). When the antisera were mixed with the blood samples of both fish, all the activities of LDH isozymes disappeared. When the antisera were mixed with the extracts from livers and kidneys of the two fish, it was found that the C band remained in both fish, but all the other bands, including two 'additional bands' of grass carp disappeared in kidney extracts (Fig. 4a and 4b).

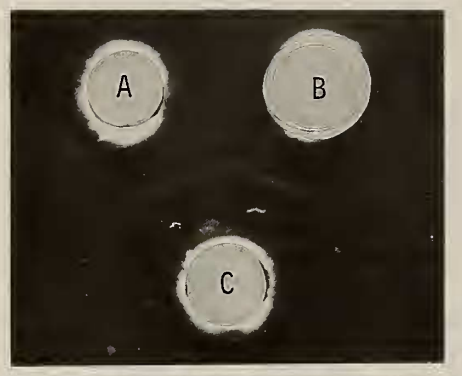

FIG. 3. Double diffusion precipitation lines of the red blood cell LDH 1–5 of Grass carp(A), and Blunt snout-bream(B) aganist the antiserum of red blood cell LDH 1–5 of Blunt snout-bream(C).

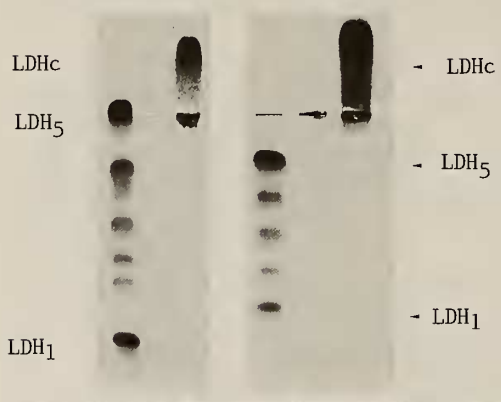

FIG. 4. Starch gel electrophoresis zymograms show, after added the antiserum of blunt snout-bream(Me) blood cell LDH 1–5 to the grass(Cp) and blunt snout-bream(Me) kidney(middle) and liver(right) extracts, the LDHc bands of liver of both fish are still remained, but all the kidney bands disappeared. Both left rows show the Ct and Me kidney LDH 1–5 zymograms without antiserum treatments. A additional bands of Ct kidney LDH isozyme are also showed by arrows.

Physico-chemical properties of LDH 1 isozyme

The starch gel electrophoresis showed that the LDH 1 isozymes of both grass carp and blunt snout-bream migrated towards the anode but they had different mobility. The LDH 1 isozyme of blunt snout-bream carried more positive charges than the grass carp LDH 1 did. The blue dextran affinity chromatography showed that both of them almost had the same affinity to the blue dextran and could be released from the blue dextran by 0.05 mg/ml of NAD. The optimum pH value, optimum temperature of reaction and denaturing concentration by urea of LDH 1 isozyme are also the same in both fish except their Km values remained different. The Km value of Grass carp LDH 1 is 1.1×10^{-1} and the km value of blunt snout-bream LDH 1 is 5.6×10^{-2}.

The amino acid content of LDH 1 of the two fish are shown in Table 2. It can be seen that some kinds of amino acid content are different in the LDH 1 isozyme of the two fish, i.e. grass carp LDH 1 has more val and blunt snout-bream LDH 1 has more lys and arg.

DISCUSSION

In vertebrates, the LDH isozymes exist as tetrad forms resulting from the random polymerization of different peptides, and having different distribution in different tissues [1]. Obviously, this different distribution is due to the different expression of genes. Comparative studies on fish LDH isozymes in earlier years were carried out mainly by the methods of electrophoresis and they only provided limited evidence for deducing its molecular structure and enzymatic properties. In this paper, some evidences obtained from the kinetic, immuno and amino acid analysis of purified LDH isozymes were observed for indicating the divergences between LDH isozymes of grass carp and blunt snout-bream.

The results of starch gel electrophoretic zymograms indicated that five LDH isozymes existed in most tissues of both grass carp and blunt snout-

TABLE 2. Amino acid contents of red blood cells LDHI of grass carp (Ct) and blunt snout-bream (Me)

Fish	LDH	Amino acid content															
		Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Len	Phe	His	Lys	Arg	Tyr
Ct	LDH 1	116	80	108	132	68	108	144	248	12	28	48	12	40	80	44	0
Me	LDH 1	132	88	124	148	80	124	164	100	12	20	28	8	44	136	92	0

bream, and in liver tissue of both fish, there existed the cathode bands-LDHc. Whitt *et al.* [14] noted that the B gene of LDH isozyme is produced by the duplication of A gene, and the C gene is produced by the duplication of B gene subsequently. Odense *et al.* [15] also found out that, in the evolution of fish, the further LDH gene duplications also existed. For example, in carp, apart from the existence of A, B and C gene loci, there were also B' and C' gene loci. But in grass carp and blunt snout-bream, it seems that a typical major LDH isozyme system encoded by A and B gene loci as well as a minor LDH isozyme system coded by a C gene locus were observed. Carp, grass carp and blunt snout-bream belong to same family, *Cyprininae*. However, as compared with carp, no B' and C' gene duplication could be found in either grass carp or blunt snout-bream. In consideration of the chromosome number differences existing in carp (2n=100), grass carp (2n=48) and blunt snout-bream (2n=48), it could be explained that the less chromosome numbers of grass carp and blunt snout-bream may decrease the possibility of gene duplications on those fish as compared with the carp. However, in the kidney tissue of grass carp, there were seven LDH isozyme bands while in the blunt snout-bream kidney tissue only five LDH isozyme bands were found. Although, at present, it is not clear how the two 'additional bands' of LDH isozyme in grass carp kidney tissue arose, we believe that some minor divergence in the LDH isozymes occurred during the divergent evolution of both fish, even though they have the same chromosome number.

The results obtained from physico-chemical properties analysis of the B gene product—LDH 1 of both fish show that they have almost the same blue dextran affinity, same optimum pH value, same optimum temperature of reaction as well as the same concentration of urea for denaturation except their Km value are different which indicates that an enzymatic property difference exists in the two fish. Table 2 also shows that some amino acid content of LDH 1 have changed in the two fish, i.e. grass carp has more val and blunt snout-bream has more lys and arg. This means that minor molecular structural differences also exist in the LDH isozymes of both fish.

The results of immuno-experiments show that the antiserum against LDH 1–5 isozymes of blunt snout-bream red blood cell not only can precipitate the red blood cell LDH 1–5 isozymes of grass carp and blunt snout-bream but also can neutralize the LDH 1–5 isozyme components of different tissues, for example, the LDH 1–5 isozymes of red blood cell, liver and kidney in both fish even including the two 'additonal bands' of grass carp kidney LDH isozyme. However, it can not neurtalize the LDH C isozyme either in grass carp or in blunt snout-bream liver tissue. It means that the LDH 1–5 isozymes, the products of A and B gene, in grass carp and blunt snout-bream have a very common immuno property and the cathod band of liver LDH isozyme components in both fish is the product of C gene [14]. It can be also proposed that the two 'additional bands' of grass carp kidney LDH 5 isozyme might be recognized as the subbands of its LDH 3 and LDH 4 components, as revealed by electrophoretic zymograms, rather than as the products of other genes, because they can also be neutralized by the antiserum against the purified LDH 1–5 isozyme of blunt snout-bream.

It can be concluded that the LDH isozymes of grass carp and blunt snout-bream have many similarities in general, but some divergeneces were observed in gene activities, molecular structures as well as some physico-chemical properties according to our above experiments. Therefore, it was confirmed that, as revealed by other authors in fish [3, 4], birds and mammals [1], the LDH isozyme might be used as a genetic maker to distinguish grass carp and blunt snout-bream in addition to their morphological criteria in taxonomy.

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REFERENCES

1 Markert, C. L. and Faulhaber, I. (1965) Lactate dehydrogenase isozyme patterns of fish. J. Exp. Zool., **159**: 319–322.

2 Shaklee, J. B., Kepes, K. L. and Whitt, G. S. (1973) Specialized lactate dehydrogenase isozyme: the molecular and genetic basis for the unique eye and liver LDHs of teleost fishes. J. Exp. Zool., **185**: 217–240.

3 Wuntch, T. and Goldberg, E. (1970) A comparative physicochemical characterization of lactated dehydrogenase isozymes in brook trout, lake trout and their hybrid splake trout. J. Exp. Zool., **174**: 233–252.

4 Yan, J., Wang G. and Yan, S. (1986) Analysis of starch gel electrophoresis patterns of hemoglobin and red blood cell LDH isozymes of four kinds of fresh water teleost (*Mylopharyngodon piceus, Ctenopharyngodon idellus, Hypophthalmichtys molitrix, Aristichys nobilis*). Hereditas (Beijing), **8**: 25–27.

5 Okabe, K., Hayakawa, Hamada, M. and Koike, M. (1968) Purification and comparative properties of human lactate dehydrogenase isozymes from uterus, uterine myoma and cervical cancer. Biochemistry, **7**: 79–90.

6 Cuatrecasas, P. (1970) Protein purification by affinity chromatography. J. Biol. Chem., **245**: 3059–3065.

7 Fulton, J. A., Schlabach, T. D., Kerl, J. E., Toren, E. and Clifford, J. (1979) Dual-detector-post-column reactor system for the detection of isozymes separated by high-performance liquid chromatography. II. Evaluation and application to lactate dehydrogenase isozymes. J. Chromatogr., **175**: 283–291.

8 Pesce, A., McKay, R. H., Stolzenback, F. R. D. and Kaplan, N. O. (1964) The comparative enzymology of lactate dehydrogenases. I. Properties of the crystalline beef and chicken enzyme. J. Biol. Chem., **239**: 1753–1761.

9 Xue, G. (1978) Lactate dehydrogenase isozyme. Shengwu Kexue Dongtai, **3**: 10–17.

10 Holmes, R. S. and Soopes, R. K. (1974) Immunochemical homologies among vertebrate lactate dehydrogenase isozymes. Eur. J. Biochem., **43**: 167–177.

11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the phenol reagent. J. Biol. Chem., **193**: 265–275.

12 Amino Acid Analysis System, Operator's Manual. (1983) Waters Associates Publications, Milford, MA, U.S.A. pp. 4–1—4–11.

13 Clausen, J. (1981) Immunochemical Techniques for the Identification and Estimation of Macromolecules. Elsevier North-Holl and Biomedical Press, Amsterdam.

14 Witt, G. S., Shaklee, J. B. and Markert, C. L. (1975) Evolution of the lactate dehydrogenase isozymes of fishes. Isozymes, **4**: 381–400.

15 Odense, P. H., Allen, T. M. and Leung, T. C. (1966) Multiple forms of lactate dehydrogenase and aspartate aminotransferase in herring (Clupea harengus L.) Can. J. Biochem., **44**: 1319–1326.